

Effects of Different Initial pH of Modified Zarrok Medium on Large-scale *Spirulina Maxima* Culture

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Abstract—The effects of Modified Zarrouk Medium (MZM) with different pH on *Spirulina maxima* culture were determined using five parameters: the growth rate, lag phase duration, nutrients (protein, carbohydrate and lipid), pigments (chlorophyll-a, betacarotene and phycocyanin), and morphology of the algae. The experiment was divided into 4 treatments. All treatment contained the same MZM; however, different initial pH of 9, 9.5, 10 and 10.5 were applied to the experimental treatment I, II, III and IV, respectively. The parameters of these treatments were compared to those of the positive control (the Zarrouk medium pH 9). The results showed that the algae grown in media treatment I could reduce the duration of lag phase and gave the highest specific growth rate (4.12 ± 0.005 g/l), while the algae grown in media treatment IV provided the lowest specific growth rate (1.09 ± 0.006 g/l). Regarding nutrients, the algae grown in media treatment I provided highest protein content of 71.0 ± 0.06 % and the algae grown in media treatment III provided the highest carbohydrate content of 17.07 ± 0.02 %. It was found that algae grown in media treatment I yielded the best in pigment contents, which were the chlorophyll-a (15.2 ± 0.16 mg/g), beta-carotene (3.16 ± 0.32 mg/g) and phycocyanin (46.37 ± 0.20 mg/g). The Morphological studies of algae using microscope SEM and TEM revealed that most trichome of algae grown in media treatment III and group IV were changed from helicoidal to straight.

Index Terms—*Spirulina maxima*, pH, nutrient, pigment, growth rate, morphology.

I. INTRODUCTION

Spirulina maxima is a Bluegreen algae that can fully thrive in tropical zones. Taken as highly valuable algae, it gains popularity among various types of industries, such as therapeutic and cosmetics industry. They use some important substances for secondary product and phycocyanin extracted from *Spirulina maxima* [1]. In medical field, it is used to prevent and cure many diseases e.g. prevention of diabetes [2] anti-inflammation [3] reduction of swelling, and resistant of Entervirus71 [4].

Moreover, *Spirulina maxima* is used in dietary supplement industry. Globally, the production of *Spirulina maxima* is 3,000 tons (dry weight) per year [5] with Mexico, Japan and USA as the top three largest producers.

In Thailand, there are only three companies with the highest production capacity, and more ten small scale producers. *Spirulina maxima* culture techniques vary from place to place based on geographical locations and producers' knowledge. Consequently, there are numerous studies on various aspects of *Spirulina maxima* cultivation as a variety of factors influence the cultivation including intensity of starter, strain, pH level, light intensity, carbon level, and temperature [6]. Regarding the essential factors stated, it is found that Thailand has favorable temperature, light intensity, and strain. Thus, further experiments have been conducted on other factors that are controllable and help to increase productivity [7]. For example, there are studies on carbon dioxide [8] and pH level as *Spirulina maxima* grows in high pH level media [9]-[11], and pH level also influence carbon dioxide and mineral dissolution as well as metabolism process in *Spirulina maxima* cells [9].

This research is a part of a producer who is willing to study the effects of Modified Zarrouk Medium at different initial pH levels which are related to growth rate, lag phase duration, necessary food, pigment, and forms of *Spirulina maxima*, in order to be applied in implantation in large scale industry and to be used as a database for gathering information of other factors influencing upbringing and yielding products with quality.

II. MATERIALS AND METHODS

A. Preparation of Stock Culture

Spirulina maxima is collected at the Institute of Research and Food Products, Kasetsart University, Bangkok, Thailand. The stock culture of *Spirulina maxima* was prepared in pond (4x15x0.2 cubic meter) using Zarrouk medium as the media. Medium was stirred using paddles and allowing sun light pass through the medium. The *Spirulina maxima* stock culture was readily to be used when the optical density reached 1.0 (OD₅₆₀).

B. Effect of pH on Cultivation of Spirulina Maxima

The *Spirulina maxima* was cultured in Modified Zarrouk Medium with different initial pH (i.e. pH 9-10.5). The pH of the medium was adjusted using NaOH solution.

C. Growth Measurement

The optical density was measured at 560 nm using spectrophotometer. The optical density was measured for everyday and the pH was measured twice a day. Algae cells were filtered using straining cloth (filter cloth). The filtered algae was dried in oven at 60 C for 6 hrs. The dried algae was weighed and recorded.

D. Analysis of Nutritional Value and Pigment

The nutritional values (i.e. protein, fat, and carbohydrate) were analyzed using the method published by Chu *et. al* [10]. The pigments (chlorophyll, carotene, phycocyanin) were determined using spectrophotometry [1], [11], [12].

E. Morphological Studies

Algae was post fixed in 0.2% OsO₄, then it was dehydrated by passage through an acetone-water series (25-50-75-100% acetone), and then dried at the critical point in liquid CO₂. All samples were viewed by scanning electron microscope (SEM), JEOL.

Trichome morphology of *Spirulina maxima* was studied by the transmission electron microscope (TEM) [13]. Algae was collected in 1-ml centrifuge tubes and then centrifuged at 1500 rpm for 20 min, followed by separate fixation in 0.1 M sucrose solution containing 4% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.0) at 4 °C for 2 hrs. They were then rinsed twice with a 0.1 M sodium cacodylate buffer containing 10 mM CaCl₂, and sucrose concentration successively reduced to 0.05 M. This treatment was followed by two rinses by 0.1 M sodium cacodylate buffer containing 10 mM CaCl₂. Post-fixation was performed with 2% OsO₄ in 0.1 M sodium cacodylate buffer containing 10 mM CaCl₂ for 1 hr at 4 °C. Thereafter, all materials were rinsed four times with a sodium cacodylate buffer containing 10 mM CaCl₂, three times with aqueous ethanol (50%) and gradually dehydrated in ethanol (40, 80, 95, 100%). Dehydrated materials were prepared for transmission electron microscope (TEM, JEOL, JEM-2100).

III. RESULTS AND DISCUSSION

A. Growth Measurement

The growth of *Spirulina maxima* in MZM at different initial pH was studied (T1, pH 9.0; T2, pH 9.5; T3, pH 10; T4, pH 10.5). Optical density measurement was used to assess *Spirulina maxima* growth. From Fig. 1, the optical density of all treatments was started at 0.2. The highest optical density was found in T1 (pH 9.0) at day 5. Exponential growth phase was found during this day. This value is not significantly different to the value obtained in T2 (optical density =0.58), but it is significantly different to T3 (optical density=0.3) and T4 (optical density=0.2). The statistically confident level at p≤0.05 was used throughout this work.

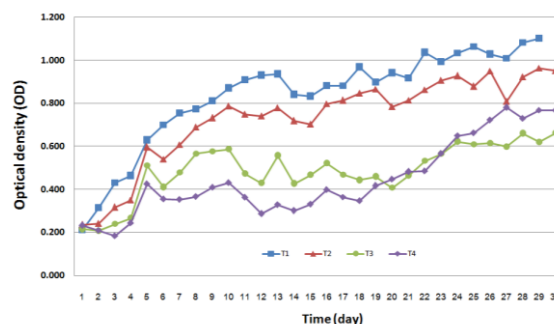


Figure 1. Optical density of modified zarrouk medium with different pH on *Spirulina maxima* culture

Considering the growth rate using term of μ_{max} (d⁻¹), the results can be shown in Table I. It was found that T1(pH9) posed highest rate at 0.40. Meanwhile, the growth rates for T2, T3 and T4 were 0.37, 0.31 and 0.03, respectively. Algae in T1 is able to reduce the lag phase to 5 days, and it also posed highest the specific growth rate (4.12±0.005 grams per liter) and protein production (71.0±0.06 percent). The lowest rate of carbohydrate production was found in T3 (pH 10). Its value was 14.70±0.02 percent.

The effect of initial pH on the production of chlorophyll, beta carotene, and phycocyanin were studied. The results obtained are shown in Table II. The highest amount of chlorophyll, beta carotene, and phycocyanin were obtained from T1 (pH 9.0). Their values were 15.2±0.16 milligrams per gram, 3.16±0.32 milligrams per gram, and 46.37±0.20 milligrams per gram, respectively.

TABLE I. GROWTH AND BIOCHEMICAL CONSTITUENTS OF SPIRULINA MAXIMA GROWN IN MODIFIED ZARROUK MEDIUM WITH DIFFERENT PH

pH level	μ_{max}^{-1} (d ⁻¹)	Dry weight (g/L)	Protein %DW	Carbohydrate % DW	Fat %DW
9	0.40	4.12±0.005 ^a	71±0.065 ^a	15.32±0.11 ^a	8.21±0.32 ^a
9.5	0.37	3.74±0.015 ^a	70±0.086 ^a	15.63±0.08 ^a	8.13±0.14 ^a
10	0.31	2.89±0.012 ^b	64.3±0.32 ^a	14.7±0.02 ^b	6.93±0.08 ^b
10.5	0.03	1.09±0.006 ^c	61.3±0.21 ^b	17.43±0.14 ^a	6.78±0.71 ^b

^{a, b, c} Considering the same alphabet, they indicate no significant differences at a 95% confidence level (calculated by Duncan Multiple Range Test)

TABLE II. CHLOROPHYLL, BETA-CAROTENE, PHYCOCYANIN OF SPIRULINA MAXIMA GROWN IN MODIFIED ZARROUK MEDIUM WITH DIFFERENT PH

pH level	Chlorophyll (mg/g)	Beta-carotene (g/g)	Phycocyanin (mg/g)
9	15.2±0.16 ^a	3.16±0.02 ^a	46.37±1.20 ^a
9.5	14.7±0.32 ^a	3.12±0.13 ^a	46.42±0.13 ^a
10	10.16±0.03 ^b	3.47±0.04 ^a	42.45±1.22 ^a
10.5	9.34 ±0.13 ^b	2.77±0.25 ^b	27.30±1.23 ^b

^{a, b, c} Considering the same alphabet, they indicate no significant differences at a 95% confidence level (calculated by Duncan Multiple Range Test)

B. Morphological Studies

Morphological studies were conducted by using microscope technique, SEM, and TEM. The result of high growth rate obtained from treatment 1 (pH 9.0) and the less growth rate obtained from treatment 4 (pH 10.5) were interesting results.

Therefore, the results from these two systems were chosen to further discussion and compare in term of its morphologies. From microscope results, the algae cells in treatment 1 (pH 9) is spiral (Fig. 2A). Meanwhile, the algae cells in treatment 4 (pH 10.5) has become unscrewed forming the line shape (Fig. 3 A). These obtained results are agreed well with the report of Kim *et al* [14] i.e. at high pH 11.5 to 12.5 the mount of spirals is decreasing and finally becoming straight line.

The 3D structures of algae obtained from treatment 1 and 4 were studied by SEM experimentation. Their shapes were totally different. The equally twisting shape was shown for algae in treatment 1 (Fig. 2B). The whole straight line shape was obtained for algae in treatment 4 (Fig. 3B).

Therefore, increasing initial pH level restrains the spiral shape. Beside the pH variation, other factors such as salinity, environment would be able to affect the transforming shape (spiral to straight) [15].

The change of internal elements of cells and membrane after pH variation was studied by TEM. For treatment 1-pH 9 (Fig. 2C), the separated septum inside algae cell and the gas vacuoles spreading around layers was observed. Meanwhile, for treatment 4-pH 10.5 (Fig. 3C), the unclear septum, less gas vacuoles in some parts and cell wall were observed.

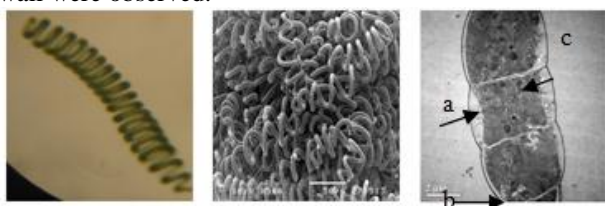


Figure 2. Morphology of *Spirulina maxima* treatment 1 (A) under microscope compound (B) by scanning electron microscope (SEM) (C) by transmission electron microscope (TEM). Arrow "a" septum, "b" thylacoid bundle and "c" gas vacuole. Scale bar =2 μ m

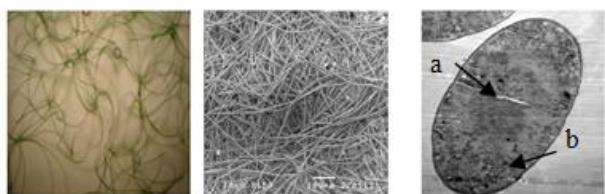


Figure 3. Morphology of *Spirulina maxima* treatment 4 (A) under microscope compound (B) by scanning electron microscope (SEM) (C) by transmission electron microscope (TEM). Arrow "a" septum and "b" thylacoid bundle. Scale bar =2 μ m

Therefore, the monitoring of cell status and the appropriate harvest time of *S. platensis* were determined using the dry cell weight, growth rate, lag phase duration, nutrient production, pigment production, and morphology of the algae. From the results obtained, it was found that

the algae cultivation in MZM at pH 9-9.5 gave the good results in term of all parameters studied as well as its morphology. In this pH, the spiral algae shape was observed. It is convenient for algae harvest (filtering through 40 μ m filter cloth).

The experimentation, determination, and data evaluation taking place in this work would be the fruitful example, and provide useful information for small and large scale cultivations of *S. maxima*. The large scale up to the industrial scale would be beneficial.

IV. CONCLUSION

The growth rate, lag phase duration, nutrients (protein, carbohydrate and lipid), pigments (chlorophyll-a, betacarotene and phycocyanin) and the morphology of *S. maxima* is strongly dependent on the pH of MZM system. Algae in T1 (pH 9) is able to reduce the lag phase to 5 days, and it also posed highest the specific growth rate (4.12 ± 0.005 grams per liter) and protein production (71.0 ± 0.06 percent). In addition, the highest amount of chlorophyll, beta carotene, and phycocyanin were obtained from T1 (pH 9.0). Their values were 15.2 ± 0.16 milligrams per gram, 3.16 ± 0.32 milligrams per gram, and 46.37 ± 0.20 milligrams per gram, respectively. From microscope and SEM monitoring, the algae cells in treatment 1 (pH 9) is spiral shape; meanwhile it is straight line at higher pH (10-10.5). The spiral shape is more convenient in algae harvesting step (filtering through 40 μ m filter cloth). From the TEM studies, it was found that the septum inside algae cell and the gas vacuoles inside algae cell were obviously dependent on the pH variation. At high pH (10-10.5), the unclear septum, less gas vacuoles in some parts and cell wall were observed. This affects to the mass ratio of wet/dry algae weights. Therefore, based on the results obtained in this work, the low pH at 9 is suitable for *S. maxima* cultivation. This result would be fruitful for the application to the large and industrial scales.

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